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Identification and functional characterization of the house finch interleukin-1β

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Highlights

- Full-length house finch IL-1β was cloned, expressed, and its basic biological roles explored.
- House finch IL-1β modulates the expression of Th1/Th2 cytokines and nitric oxide production by activated immune cells.
- House finch IL-1β enhances the expression of acute phase protein and antimicrobial peptide by activated immune cells.

Abstract

Interleukin-1β (IL-1β), an inflammatory cytokine of the IL-1 family, is primarily produced as a precursor protein by monocytes and macrophages, then matures and becomes activated through proteolytic catalysis. Although the biological characteristics of avian IL-1β are well known, little information is available about its biological role in songbird species such as house finches that are vulnerable to naturally-occurring inflammatory diseases. In this study, house finch IL-1β (HfIL-1β) was cloned, expressed, and its biological function examined. Both precursor and mature forms of HfIL-1β consisting of 269 and 162 amino acids, respectively, were amplified from total RNA of spleen and cloned into expression vectors. HfIL-1β showed high sequential and tertiary structural similarity to chicken homologue that allowed detection of the expressed mature recombinant HfIL-1β (rHfIL-1β) with anti-ChIL-1β antibody by immunoblot analysis. For further characterization, we used primary splenocytes and hepatocytes that are predominant sources of IL-1β upon stimulation, as well as suitable targets to stimulation by IL-1β. Isolated house finch splenocytes were stimulated with rHfIL-1β in the presence and absence of concanavalin A (Con A), RNA was extracted and transcript levels of Th1/Th2 cytokines and a chemokine were measured by qRT-PCR. The addition of rHfIL-1β induced significant enhancement of IL-2 transcript, a Th1 cytokine, while transcription of IL-1β and the Th2 cytokine IL-10 was slightly enhanced by rHfIL-1β treatment. rHfIL-1β also led to elevated levels of the chemokine CXCL1 and nitric oxide production regardless of co-stimulation with Con A. In addition, the production of the acute phase protein serum amyloid A and the antimicrobial peptide LEAP2 was observed in HfIL-1β-stimulated hepatocytes. Taken together, these observations revealed the basic functions of HfIL-1β including the stimulatory effect on cell proliferation, production of Th1/Th2 cytokines and acute phase proteins by immune cells,

thus providing valuable insight into how HfIL-1β is involved in regulating inflammatory response.

Keywords: IL-1β; house finch; cytokines; acute phase protein; avian

1. Introduction

Interleukin-1beta (IL-1β) is the most studied prototypical pro-inflammatory cytokine because of its crucial role in the initiation of inflammation and regulation of innate and adaptive immune responses (Netea et al., 2015). IL-1β lacks a signal peptide and is primarily expressed by activated macrophages, monocytes, and dendritic cells as an inactive precursor form and remains in the cytosol, requiring proteolytic processing at its N-terminal region for optimal bioactivity (Black et al., 1988; Thornberry et al., 1992; Arend et al., 2008). Subsequently, it is cleaved by either an intracellular cysteine protease caspase-1 activated by inflammasome (Thornberry et al., 1992; Martinon et al., 2002) or by inflammasome-independent enzymatic processes such as neutrophil-derived serine proteases and pathogen-released enzymes (Netea et al., 2010). This cleaved IL-1β is secreted into the extracellular milieu, where it can induce its own transcription as mature and bioactive IL-1β. By binding to IL-1 type I receptor (IL-1R1), secreted IL-1β exerts its biological activities including T cell activation, B cell proliferation, and antigen recognition along with the induction of inflammatory genes, chemokines, and cell adhesion molecules (Burns et al., 2003; Dinarello, 2009). In mammals, IL-1β induces the development of Th17 cells in combination with IL-6 or TGF-β, while the production of IL-23 is IL-1β dependent in monocytes which contributes to maintenance of Th17 cells (Weaver et al., 2007; Dong, 2008; van de Veerdonk et al., 2009). IL-1β also induces synthesis of cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and inducible nitric oxide synthase (iNOS), leading to the production of prostaglandin-E2 (PGE2), platelet activating factor (PAF), and nitric oxide (NO) that causes fever, lower pain threshold, vasodilatation, and hypotension (Dinarello, 2009). Additionally, IL-1β is responsible for triggering the synthesis of the acute phase protein serum amyloid A (SAA), IL-6, neutrophil-selective CXC chemokines, and

72 macrophage inflammatory protein-2 (McColl et al., 2007). An abnormal increase of IL-1β
73 secretion is associated with the pathogenesis of auto-inflammatory diseases such as cryopyrin-
74 associated periodic syndromes, which is related to an over-activation of caspase-1 (Campbell et
75 al., 2016).

76 In avian species, chicken IL-1β (ChIL-1β) was first identified and cloned from the
77 chicken macrophage cell line HD11 stimulated with LPS (Weining et al., 1998). ChIL-1β has a
78 similar gene structure to mammalian homologues (Giansanti et al., 2006) with 34% and 33%
79 amino acid identity with the respective human and mouse orthologues; however, it lacks a
80 conserved aspartic acid residue thus preventing the caspase-1 cleavage. Nonetheless, N-
81 terminally truncated ChIL-1β lacking the predicted pro-domain exhibits significantly enhanced
82 biological activity suggesting that precursor cleavage is critical for its maximal activity (Gyorfy
83 et al., 2003). Another phylogenetically conserved aspartic acid residue was later discovered by
84 cleavage of avian proIL-1β with either sea bass or human caspase-1, which is distinct from the
85 cleavage site of mammalian homologues (Reis et al., 2012). Consistent with mammalian
86 homologues, ChIL-1β expression is significantly enhanced following viral, bacterial, and
87 protozoal infections. ChIL-1β mRNA expression was induced in the gut following *Eimeria*
88 infection (Laurent et al., 2001; Hong et al., 2006a,b), enhanced mRNA level was also observed
89 in macrophages from turkeys suffering from poult enteritis and mortality syndrome (PEMS), as
90 well as in bursal cells from IBDV-infected chickens (Heggen et al., 2000; Eldaghayes et al.,
91 2006). *Salmonella* spp. led to up-regulation of IL-1β mRNA in chicken cell lines and heterophils
92 (Iqbal et al., 2005; Kogut et al., 2005). Macrophages exposed to either *Escherichia coli* or
93 *Mycoplasma synoviae* increased IL-1β transcription (Lavric et al., 2008). These reports further
94 highlight the important role of IL-1β in controlling the pathogenesis of many diseases.

The properties of IL-1β have been well studied in domestic poultry but not in wild birds, which are in close contact with domesticated animals and may act as natural reservoirs for many zoonotic pathogens. The house finch, *Haemorhous mexicanus*, is a small passerine songbird that originally inhabited western North America and later expanded to the eastern U.S. (Hill, 1993). House finches are relatively easy to capture and examine in captivity making them ideal organisms for studying the ecology of wildlife diseases, and they favored over domesticated birds to study the co-evolutionary relationship between host and pathogen during emergence of other diseases (Hurtado, 2012). Most recently, differential mRNA expression of IL-1β across populations following experimental *Mycoplasma gallisepticum* (MG) infection was documented (Adelman et al., 2013). However, the biological role of IL-1β in wild house finches still needs to be elucidated. To clarify this matter, we first cloned the precursor and mature forms of house finch IL-1β (HfIL-1β), then investigated its basic function by measuring immune cell proliferation and differential mRNA expression of Th1/Th2 response elements, acute phase protein and antimicrobial peptide by activated immune cells.

2. Materials and Methods

2.1. Birds and tissue collection

House finches were captured in either July of 2012 or June-July of 2015 using cage traps and mist nets in Montgomery County, VA under permits from VDGIF (044569/2012 and 050352/2015) and USFWS (MB158404-1). All finches were housed at constant day length and temperature, and were fed an *ad libitum* pelleted diet prior to and throughout experiments (Daily Maintenance Diet, Roudybush Inc. Woodland, CA). Following capture, adult individuals from both sexes were identified based on their plumage characteristics and tested for the exposure to

the pathogen as described in Park et al. (Data in Brief, submitted). After testing, only healthy birds that showed no clinical signs of disease and had no pathogen load (Grodio et al., 2008) were randomly selected for the subsequent experiments. All tissue samples, including brain, heart, liver, small intestines (duodenum, jejunum, ileum), spleen, thymus, bursa, lung, proventriculus and gizzard were collected from two individuals to assess HfIL-1β tissue distribution. Additionally, the primary cells were isolated from spleens and livers of 10 randomly selected birds for further biological experiments.

2.2. Sequence and structural analyses

Nucleotide and amino acid sequences of HfIL-1β were aligned with other orthologous sequences obtained by BLAST search using Clustal Omega (Sievers and Higgins, 2014). The phylogenetic tree was constructed from the alignment using the neighbor joining (NJ) method within the MEGA4 program, with Poisson correction and complete deletion of gaps (Tamura et al., 2007). The stability of the branching order was confirmed by performing 1,000 bootstrap replicates. The theoretical molecular weight (MW) and isoelectric point (pI) were estimated using a Compute pI/MW tool from ExPASy (http://www.expasy.org). The three-dimensional structure of HfIL-1β was built by comparative modeling at the Robetta server (http://robetta.bakerlab.org) (Kim et al., 2004). The model was superimposed with the X-ray structure of ChIL-1β using Discovery Studio 2.0 (Accelrys Inc., CA) and PyMOL (DeLano Scientific, CA).

2.3. Construction of recombinant HfIL-1β (rHfIL-1β) expression plasmid

Both precursor and mature forms of HfIL-1β genes were amplified from total RNA extracted from house finch spleen using the primers designed based on partial genomic sequences of house finch (provided by D. Hawley) (Table 1). Using 1 μg of total RNA, the first-strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, CA). The full-length HfIL-1β was amplified using the following conditions: initial denaturation at 92°C for 2 min, 35 cycles of denaturation at 92°C for 15 sec, annealing at 54°C for 15 sec and extension at 72°C for 30 sec, with a final extension at 72°C for 7 min. Synthesized precursor and mature forms of HfIL-1β were directly inserted into pCR2.1-TOPO vector (Invitrogen, CA) and transformed into *E. coli* TOP10 (Invitrogen). Transformants containing recombinant plasmid were selected by a combination of PCR screening and endonuclease digestion with *EcoR* I (New England Biolabs, MA), and confirmed by sequencing (Biocomplexity Institute at Virginia Tech, VA). For sub-cloning into a prokaryotic or eukaryotic expression vector, mature and precursor forms of HfIL-1β were digested with endonucleases *Bgl* II and *Xma* I (New England Biolabs) and ligated into pQE-30 (Novagen, CA) and pcDNA3.1 (Invitrogen), respectively. By colony PCR screening, positive clones including HfIL-1β were selected and verified by sequencing.

2.4. Expression of rHfIL-1β and immunoblot analysis

HfIL-1β in pQE30 plasmid was introduced into *E. coli* BL21 (New England Biolabs) and cultured at 30°C overnight. The expression of HfIL-1β was induced by adding 1 mM IPTG (Gold Biotechnology, MO) and shaking incubation for 5 hr at 25°C. The cells were harvested by centrifugation and resuspended with 50 mM Tris (pH 7.5), 240 mM NaCl and 1 mg/ml lysozyme buffer. After cell lysis by sonication, soluble fraction containing HfIL-1β was collected by centrifugation, followed by purification using Ni^{+}-resin (Bioline, MA). After endotoxin removal

using the ProteoSpin Endotoxin Removal Micro Kit (Norgenbiotek, ON, Canada), the purified rHfIL-1β was quantified using BCA protein assay and used in subsequent assays. To examine the binding reactivity of anti-ChIL-1β antibody, 1 μg of the purified rHfIL-1β, and rChIL-1β (Bio-Rad) as a positive control were loaded on SDS-PAGE gel under reducing conditions and transferred to PVDF membrane (Millipore, MA). The blot was incubated with anti-polyhistidine conjugated with HRP (Sigma, MO) or anti-ChIL-1β polyclonal antibody (Thermo Scientific, MA) in a 1: 1,000 dilution as the primary antibody and goat anti-rabbit IgG conjugated with HRP (Santa Cruz Biotechnology, CA) in a 1: 2,000 dilution as the secondary antibody. After washing, the blot was incubated with the SuperSignal West Pico chemiluminescent Substrate (Pierce, IL), and developed using a gel imaging system (Bio-Rad).

2.5. HfIL-1β expression analysis in tissue

The expression of HfIL-1β in house finch tissues was determined by qRT-PCR and immunoblotting. In order to investigate HfIL-1β mRNA expression, various tissues were collected from two healthy house finches including brain, heart, liver, spleen, thymus, bursa, lung, proventriculus, gizzard and each small intestinal section. Total RNA was extracted using RNeasy Mini Kit (Qiagen, CA), followed by synthesis of the first-strand cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). Synthesized cDNA was mixed with 5 μl of Fast SYBR Green Master Mix (Applied Biosystems) and 0.1 μM primers in 10 μl final volume of qRT-PCR reaction. The following thermal cycling conditions were used: 95°C for 20 sec as initial denaturation, followed by 40 cycles of denaturation at 95°C for 3 sec, and annealing/extension at 57°C for 30 sec. Transcription of HfIL-1β was normalized against the expression of GAPDH, followed by calibration using brain transcript level and $2^{-\Delta\Delta CT}$ method

(Livak and Schmittgen, 2001). To examine HfIL-1β protein expression level, 50 mg of tissues were collected from same birds that we used for RNA extraction were homogenized and sonicated in RIPA buffer (Cayman Chemical, MI) supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3Vo_4). After centrifugation at 10,000 x *g* for 30 min, the supernatant was collected and protein concentration determined using BCA assay (Thermo Scientific), then a 20 μg protein extract was resolved on SDS-PAGE gel under reducing conditions and analyzed by immunoblotting with anti-ChIL-1β antibody as previously described. In parallel, anti-GAPDH antibody (1:4,000; Millipore) was used as a reference for protein loading and for quantification of relative protein expression.

2.6. Isolation of splenocytes and hepatocytes

To isolate splenocytes, house finch spleens were excised and passed through a 0.22 μm cell strainer (BD, CA). Cell debris was washed out of cell suspension with Hank's Salt Solution (HBSS; HyClone, UT), which was overlaid onto Histopaque-1077 (Sigma). After centrifugation at 400 x *g* for 30 min, mononuclear cells from the interphase were collected and mixed with PBS. By centrifugation, cells were collected and washed with RPMI-1640 (Mediatech, VA), and counted using a hemocytometer. Freshly isolated splenocytes were resuspended with RPMI-1640 containing 20% fetal calf serum (FCS; Atlanta Biologicals, GA) and 1% penicillin/streptomycin, and cultured in a 24-well plate at a cell density of 1×10^6 cells/well overnight at 39°C with 5% CO_2 humidified air. For the isolation of hepatocytes, the livers were excised and cut into small pieces. After washing with HBSS, the pieces were incubated with 0.25% trypsin in Dulbecco's Modified Eagle Medium (DMEM; Mediatech) for 18 hr at 4°C and then placed at 37°C for 30 min. The tissue pieces were passed through a 0.22 μm cell strainer

and the collected cells were washed with DMEM. After determining cell viability and concentration by a hemocytometer, the cells were resuspended with DMEM supplemented with 10% FCS and 1% penicillin/streptomycin and seeded at 1×10^6 cells/well in a 24-well plate and then cultured overnight at 39°C with 5% CO_2 humidified air.

2.7. Cell proliferation assay

The role of HfIL-1β on cellular proliferation was investigated with either splenocytes or hepatocytes using CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega, WI) according to manufacturer's protocol. Briefly, 2×10^5 cells were seeded in a 96-well plate and incubated with medium alone, rHfIL-1β (0.01 and 0.1 μg/ml) with or without Con A in the presence and absence of anti-ChIL-1β antibody at 39°C with 5% CO_2 for 12 hr. Incubated cells were treated with Dye Solution (15 μl) for 3 hr at 39°C with 5% CO_2, followed by addition of Solubilization Solution/Stop Mix. After 1 hr incubation at 39°C, the absorbance was measured at 570 nm and 630 nm using a microplate reader. The readings were corrected by subtracting the background value at 630 nm.

2.8. Cytokine transcripts analysis upon cell stimulation

Isolated splenocytes (1×10^6 cells/well) were cultured in a 24-well plates and treated with medium alone, Con A (10 μg/ml), rHfIL-1β (0.1 μg/ml), or rHfIL-1β (0.1 μg/ml) with Con A (10 μg/ml) for 6 and 12 hr. Cell supernatants were collected for quantification of NO production and total RNA was extracted from the treated cells using RNeasy Mini Kit (Qiagen). Extracted RNA (1 μg) was reverse transcribed into cDNA using High-Capacity cDNA reverse transcript kit (Applied Biosystems). The transcript levels of Th1/Th2 cytokines (IFN-γ, IL-1β, IL-2, IL-10),

iNOS and a chemokine (CXCL1) were measured by qRT-PCR. The primers used for qRT-PCR analysis were designed within the conserved regions of the multiple sequence alignment of closely-related bird species including zebra finch, canary, and chicken. To measure hepatic gene expression by HfIL-1β treatment, isolated liver hepatic cells (1×10^5 cells/well) were cultured in 24-well plates, followed by treatment with medium alone, Con A (10 μg/ml), rHfIL-1β (0.01 and 0.1 μg/ml) or rHfIL-1β (0.01 and 0.1 μg/ml) with Con A for 6 hr. After incubation, the cell supernatants were collected to measure NO production and total RNA was extracted as described earlier. The mRNA levels of the SAA, LEAP2, and IL-1β were analyzed. With the collected cell supernatants, NO production was measured using a Griess Reagent System (Promega).

2.9. Statistical analyses

All data were expressed as the means ± SEM and analyzed by Student's t test or one-way ANOVA using JMP software (Ver 11). Differences between groups assessed by Tukey Kramer multiple comparison test were considered to be statistically significant at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

3. Results

3.1. Sequence analyses of HfIL-1β

The full-length HfIL-1β was predicted to encode a precursor form of 269 amino acids with a theoretical molecular weight of 30 kDa and isoelectric point of 6.74. Multiple sequence alignment of the deduced amino acid sequence with other orthologs revealed that the precursor form of HfIL-1β shares 76% and 94% similarity with chicken and zebra finch, respectively, while it has 28% and 27% similarity with human and mouse, respectively. Sequence comparison

revealed that HfIL-1β lacks the conserved aspartic acid (Asp/D) as the IL-1β-converting enzyme (ICE) cut site, and its mature form starts alanine at amino acid residue 108 producing a 162 amino acid peptide with a predicted molecular weight of 18 kDa and isoelectric point of 8.52. This mature form has 32% and 34% similarity with the respective human and mouse sequences, and 84% and 97% similarity with the chicken and zebra finch, respectively. Phylogenetic analysis indicated that the IL-1β encoding region evolved into two distinct lineages among avian species and HfIL-1β being evolutionary closer to zebra finch and pigeon IL-1βs than to that of any domestic avian including chicken, turkey, duck, goose and quail (Figure 1A). Computational analysis revealed that HfIL-1β retains six cysteine residues, and Cys^{25} and Cys^{27} as well as Cys^{187} and Cys^{241} are predicted to form disulfide bonds. The crystal structure of HfIL-1β revealed 15 β-strands and an α-helix. The house finch and chicken IL-1β (PDB entry, 2wry) structures share a very similar structural fold with a root mean square deviation (RMSD) of 0.53 Å (Figure 1B). Based on high level of sequential and structural identity between house finch and chicken IL-1β, we predicted that cross-reactivity would exist based on the anti-ChIL-1β antibody used in further biological assays.

3.2. Immunoblot analysis of rHfIL-1β

For biological function characterization, rHfIL-1β with a polyhistidine tag fused at the N-terminus was purified from *E. coli* BL21 as a soluble form. The endotoxin concentration was 0.07 endotoxin units (EU) per μg protein, which was acceptable for further cellular assay. Prior to the initiation of the biological assays, purified rHfIL-1β was confirmed by immunoblot analysis using anti-polyhistidine antibody as well as verified binding reactivity of anti-ChIL-1β antibody made against rHfIL-1β. As shown in Figure 2A, two bands were detected

approximately 19 kDa, the predicted size of HfIL-1β containing polyhistidine tag (1.1 kDa) along with 25 kDa using anti-polyhistidine antibody under reducing conditions. Blotting with anti-ChIL-1β antibody resulted in a single 25 kDa of rHfIL-1β, which is higher than the calculated size but identical to that of rChIL-1β (positive control), which is the mature form containing a polyhistidine tag expressed from *E. coli* under the same conditions (Figure 2B).

3.3. Tissue distribution of HfIL-1β

The relative abundance of HfIL-1β in tissues was examined at the mRNA and protein levels using qRT-PCR and immunoblotting, respectively. The mRNA expression of HfIL-1β was normalized to transcript of GAPDH as an endogenous reference gene and calculated as a fold change relative to the lowest level of brain (arbitrarily set at 1.0). HfIL-1β was expressed at varying levels in all tested tissues with the highest expression in the lung and proventriculus and the lowest level in the brain and heart (Figure 3A). Since mRNA expression does not necessarily predict protein expression, tissue-specific expression pattern of HfIL-1β proteins was determined by immunoblotting using anti-ChIL-1β antibody (Figure 3B). Prominent expression of HfIL-1β protein was observed approximately 35 kDa in the liver, bursa and gizzard, which is slightly higher molecular weight than the theoretical size of precursor HfIL-1β of 30 kDa. Also, less intense bands are shown in the lung and proventriculus; however, no such band was detected in the brain, which is consistent with its lowest mRNA expression. In addition to the 35 kDa band, a very weak 60 kDa band was observed in the gizzard (data not shown).

3.4. Effect of HfIL-1β on cell proliferation

The proliferative effects of the HfIL-1β on primary cells were investigated, resulting in a small but statistically significant induction of splenocyte proliferation following treatment with 0.01 μg/ml rHfIL-1β for 12 hr (Figure 4), although there was no significant difference after 24 hr (data not shown). The enhanced splenocyte proliferation was abolished when adding anti-ChIL-1β antibody thus neutralizing HfIL-1β; in contrast, control IgG had no effect. However, co-stimulation of HfIL-1β with Con A had a negligible effect on splenocyte proliferation relative to Con A alone (data not shown). Contrary to splenocytes, there was no significant proliferation in HfIL-1β-stimulated hepatocytes.

3.5. Modulation of gene expression and nitric oxide production by HfIL-1β in splenocytes

The effect of HfIL-1β on Th1/Th2 cytokine expression was evaluated in splenocytes stimulated with rHfIL-1β for 6 and 12 hr (Figure 5). The most pronounced induction of cytokine expression was shown at 12 hr post-stimulation. Treatment with HfIL-1β alone enhanced its own gene transcription by > 2 fold. Of the Th1 cytokines, IL-2 was remarkably increased approximately 383-fold by addition of rHfIL-1β compared to Con A alone, while no significant difference of IFN-γ expression was observed. The addition of rHfIL-1β alone induced IL-10 production, a Th2 cytokine. rHfIL-1β also led to elevated iNOS level (3-fold), irrespective of Con A stimulation. Up-regulation of iNOS mRNA expression results in the production of NO by splenocytes treated with 0.1 μg/ml rHfIL-1β both in the absence and presence of Con A for 6 hr (Figure 6) and 12 hr (data not shown). Transcription of chemokine CXCL1 was increased 10-fold and 18-fold in the presence and absence of Con A stimulation, respectively.

3.6. Expression of SAA, LEAP2, and IL-1β in hepatocytes

Induction of an acute phase protein (SAA) and an antimicrobial peptide (LEAP2) in hepatocytes stimulated with HfIL-1β was observed (Figure 7). HfIL-1β treatment induced transcripts of SAA and LEAP2 by incubation with 0.1 and 0.01 μg/ml rHfIL-1β, respectively, both in the presence and absence of Con A. The transcription of SAA was enhanced 2.2-fold, which was further enhanced by 8.8-fold in the presence of Con A. In contrast to the induction of IL-1β in stimulated splenocytes, IL-1β transcription was not changed in hepatocytes. With induction of acute phase and antimicrobial responses, significant production of NO by hepatocytes was observed when treated with 0.1μg/ml rHfIL-1β both in the presence and absence of Con A (Figure 6).

4. Discussion

Although many reports described the potency of IL-1β in immune responses following viral, bacterial, and protozoal infections, little is known regarding the role of house finch IL-1β in the host immune system. In this study, we identified and cloned the full-length HfIL-1β from house finch spleen and demonstrated the biological functions of its active form. Phylogenetic analysis revealed the evolutionary relationships among avian IL-1βs where HfIL-1β clustered with homologues of flying birds (zebra finch and pigeon), while separated from that of land-based birds (chicken, turkey, and quail) as well as waterfowl (duck and goose). Despite considerable phylogenetic distance between house finch and chicken IL-1β in the avian clade the tertiary structure of HfIL-1β was highly similar to that of ChIL-1β, with the β-strands and α-helix located in almost identical regions. Sequence analysis revealed that HfIL-1β lacks the aspartic acid residue that is critical to form active HfIL-1β as a result of proteolytic cleavage, but retains conserved alanine at position 108 that represents the initial residue for expression of mature form similar to other avian IL-1βs (Wu et al., 2007). These high sequential and structural identities

suggest that HfIL-1β is likely cross-reactive with anti-ChIL-1β antibody as substantiated by immunoblot analysis showing that chicken-specific antibody recognized HfIL-1β. Purified rHfIL-1β was detected at higher molecular weight (25 kDa) than its theoretical value (19 kDa) which could be caused by unfolding in the presence of a reducing agent. Such unfolding under reducing conditions would be expected from the potential intra-chain disulfide bond formed between Cys^{187} and Cys^{241} that is likely to be predominantly detected by anti-ChIL-1β antibody. Since precursor HfIL-1β contains two potential disulfide bonds, Cys^{25} and Cys^{27} as well as Cys^{187} and Cys^{241}, its molecular weight would be higher than calculated under reducing conditions, as 35 kDa shown in Figure 3. This possibility has been experimentally confirmed by detecting the expected size of mature HfIL-1β (19 kDa) in the absence of a reducing reagent (data not shown), which is consistent with earlier studies reporting that disulfide bond of murine IL-1β resulted in varying gel mobility depending on the presence of a reducing reagent (Gunther et al., 1991).

IL-1β is primarily produced by monocytes, macrophages, and dendritic cells as well as B lymphocytes and natural killer (NK) cells in low amounts. Due to an instability element in the coding region of IL-1β, mRNA would be poorly translated into protein (Bufler et al., 2004). The present study showed that HfIL-1β is expressed in a broad range of tissues, mainly in the digestive tract (proventriculus, gizzard, duodenum, and ileum), immune tissues (liver, bursa, and spleen) and respiratory (lung) tract. However, the levels of IL-1β mRNA expression were not congruent with changes of its protein production similar to previous reports of human IL-1β (Schindler et al., 1990a). LPS rapidly increased IL-1β transcript for a short time while the administration of IL-1β itself sustained its own production long term (Schindler et al., 1990b). Although we did not measure its continuous production through 24 hr, a small but significant

induction of IL-1β transcript was observed in splenocytes stimulated with HfIL-1β alone at 12 hr post-stimulation, but not with Con A. These results are in accordance with a relatively short half-life of IL-1β mRNA and a rate-limiting step in the processing of IL-1β to prevent its continuous and overwhelming activation which would result in deleterious effect on the host.

IL-1β is involved in a variety of cellular activities as both a growth factor for B cell proliferation and stimulator for the generation of Th17 cells which also co-stimulate T cell proliferation (Dinarello, 2009). Accordingly, we observed the effect of a low concentration of HfIL-1β (0.01 μg/ml) in promoting the proliferation of splenocytes in vitro. Whereas TNF-α and IL-6 are important factors in the priming phase of liver regeneration, IL-1β is known to be a potent inhibitor of liver regeneration and hepatocyte proliferation (Sparna et al., 2010). In contrast to previous reports, we did not observe significant changes with proliferation of hepatocytes after culture with HfIL-1β. Further work is needed to elucidate the regulatory function of HfIL-1β in the proliferation and regeneration of hepatocytes which may reveal the role that IL-1β plays in the pathogenesis of acute inflammatory liver injuries.

Through high affinity interaction with cell surface receptor, IL-1β induces Th1 adaptive cellular responses and triggers the production of acute phase proteins as well as other pro-inflammatory cytokines (Dinarello, 1996; 1999; Chung et al., 2009). In the current study, production of the Th1 cytokine IL-2 was elevated by HfIL-1β treatment in Con A-stimulated splenocytes indicating that it would stimulate T cell proliferation in conjunction with IL-2 release (Schultz, 1987). Previous studies have shown that IL-1β inhibits IL-10 production by memory T cells in vitro and in vivo while IL-10 counter-regulates the action of IL-1β (Zielinski et al., 2012). Contrary to previous findings, transcript of IL-10 was enhanced following stimulation with HfIL-1β which may indirectly occur via PGE2 production by IL-1β (Benbernou

et al., 1997). This induction of IL-10 transcript may consequently result in B cell proliferation and antibody production (Itoh and Hirohata, 1995). Enhancement of iNOS was observed after HfIL-1β treatment both in the presence and absence of Con A, which is also associated with PGE2 activation (Benbernou et al., 1997). Further, increased NO production was accompanied by the expression of iNOS mRNA. In accordance with previous findings (Nogawa et al., 1998), NO produced by iNOS may not only modulate the formation of PGE2, but also enhance COX-1 activity thereby facilitating the development of fever as well as acting as a mediator of inflammation. These data are indicative of the molecular mechanisms that regulate the balance in the expression of Th1 and Th2 cytokines providing the fundamental aspects of the immune response of wild birds. Consistent with previous findings where ChIL-1β stimulation induced the expression of CXCL1 in a dose-dependent manner in the chicken fibroblast cell line CEC-32 (Weining et al., 1998), chemokine CXCL1 was markedly upregulated regardless of Con A stimulation which is able to attract neutrophils and lymphocytes thereby contributing to inflammatory processes (Batra et al., 2012).

The administration of HfIL-1β also augmented the production of acute phase protein in hepatocytes, similar to previous reports demonstrating that IL-1β, IL-6, and TNF-α circulate to the liver and induce an acute phase response which is a systemic inflammatory reaction to disrupt the host's homeostasis (Gabay and Kushner, 1999; Bresnahan and Tanumihardjo, 2014). Interestingly, the expression of antimicrobial peptides (AMPs) is generally regulated by inflammatory factors such as IL-1β, TNF-α and LPS (Bando et al., 2007). Of the AMPs, LEAP2 (liver expressed antimicrobial peptide-2) was initially described to be predominantly produced in the liver and inhibited bacteria and fungi in vitro (Krause et al., 2003). In our study, LEAP2 was upregulated by HfIL-1β-stimulated hepatocytes suggesting that HfIL-1β modulates the

expression of LEAP2 directly or indirectly thus perhaps controlling innate cellular immunity. In addition, our data corroborate previous findings demonstrating that IL-1β is a major component of NO production by hepatocytes (Kitade et al., 1996).

In addition to the gene expression profiling of HfIL-1β-stimulated immune cells, the expression pattern of IL-1β in the sera following infection with MG is provided (Park et al., Data in Brief, submitted). Based on the previously reported data regarding up-regulation of IL-1β mRNA expression after MG infection, IL-1β production would be an expected pro-inflammatory response to the pathogenesis of MG infection. However, IL-1β mRNA expression levels do not necessarily reflect the secretion of biologically active protein. The data (Figure 1. in Park et al., submitted) revealed two forms (35 and 60 kDa) of putative precursor IL-1βs in sera of control birds, while more intense bands (25 and 60 kDa), possibly representing mature and dimeric precursor of IL-1βs, appeared in sera of MG-infected birds. These results raise the question of how precursor IL-1β is secreted in the blood. Although precursor IL-1β remains primarily cytosolic and its cleavage is an obligatory step to release precursor IL-1β in the extracellular milieu, the precursor IL-1β can also be released into extracellular space independent of processing by enzymes in the presence of some ICE inhibitors (Chin and Kostura, 1993). Given the elevated production of IL-1β as well as secretion of its bioactive form after MG infection, these data indicate that IL-1β may be a key cytokine in the pathogenesis of the inflammatory response and in mediation of host immune responses against MG in house finches. In this context, further investigation regarding the processing mechanisms leading to the production of active HfIL-1β and associated enzymatic counterparts that are relevant to the pathogenesis of MG infection is necessary.

In conclusion, we cloned and expressed HfIL-1β, and explored its basic functions including proliferative effect on splenocytes and hepatocytes, differential mRNA expression profiles of not only Th1/Th2 cytokines and chemokine but also acute phase protein and antimicrobial peptide by activated immune cells. Furthermore, the additional data extend previous findings by demonstrating that up-regulation of IL-1β mRNA expression after MG infection is accompanied by the bioactive form of IL-1β. Collectively, this study will help us to better understand the functional role of HfIL-1β in the host immune response along with its biological importance in the inflammatory response of wild birds against MG infection.

Acknowledgements

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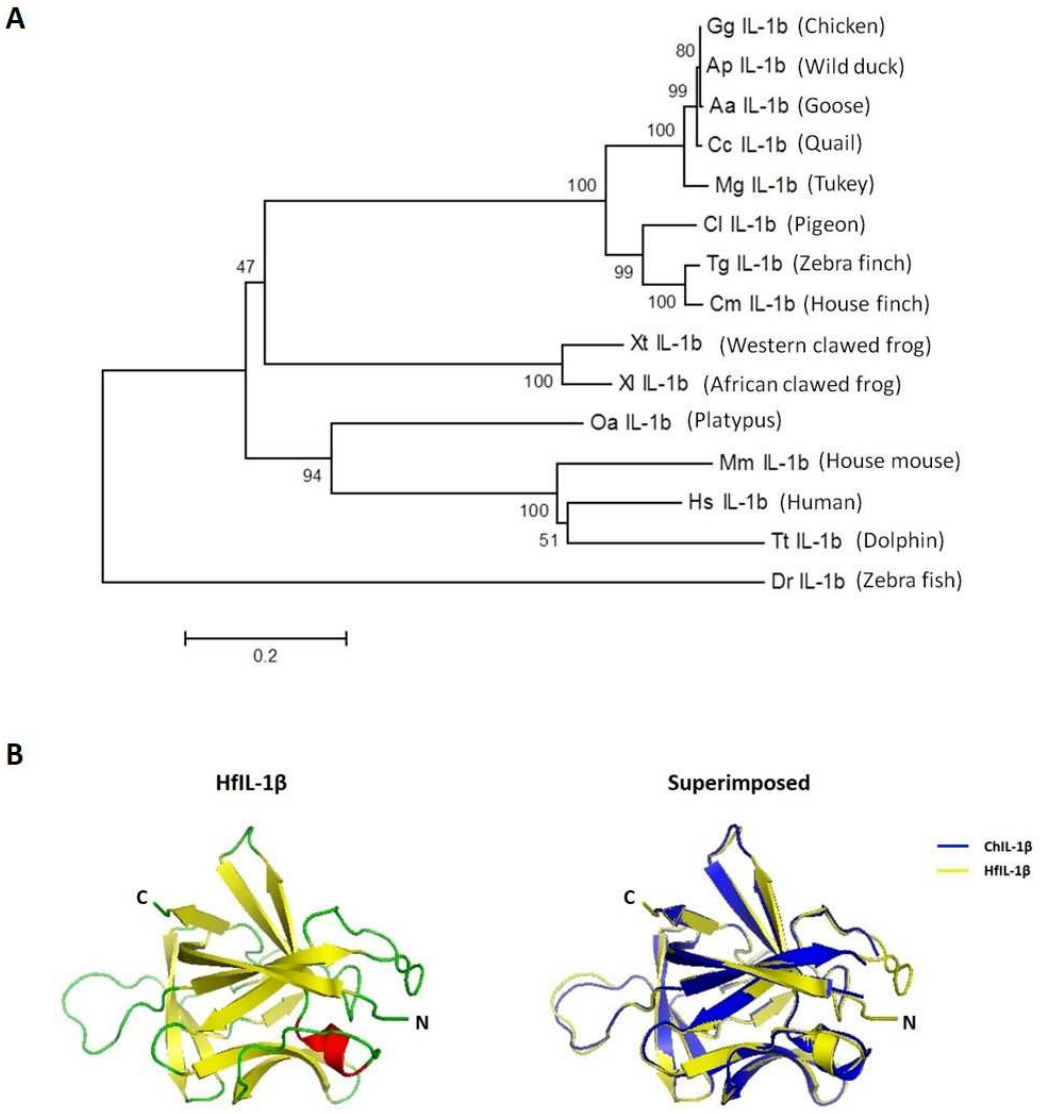
600 **Table 1.** Primers used for gene cloning and qRT-PCR analysis.

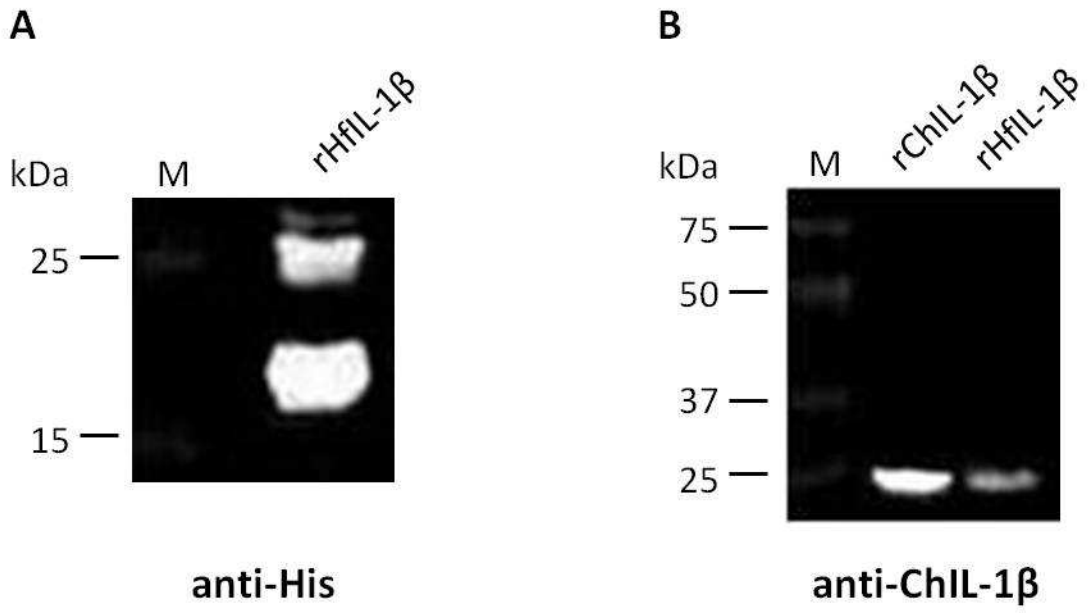
Primer Name	Nucleotide Sequence (5'→3')	Application
pHfIL-1β_F	AGATCTATGGCATTTGTCCCTGATTTGGAC	Gene cloning
mHfIL-1β_F	AGATCTGCACCTGTTTTCCGCTACACT	
HfIL-1β_R	CCCGGGTCAGCGCCCACTCAGCTCATA	qRT-PCR
IL-1β_F	GGAGGAAGCTGACATCAG	
IL-1β_R	TGTCCAGGCGGTAAAAGATG	
IFN-gamma_F	CAAAGGACCATGTCAGGAACA	
IFN-gamma_R	TGAGCCATCAGAAAGGTTTGC	
IL-2_F	TCTTGACTTTTACACACCGAATGAC	
IL-2_R	TCCTCCTCTTCCACATCTTGTTTC	
IL-10_F	AGCACCAGCGCAGCATGA	
IL-10_R	TCATCGTGGCTCTCAGGTTCA	
iNOS_F	TGCCACAAACAATGGTAATATAAGG	
iNOS_R	TGTTCCACACACGGAAATCG	
CXCL1_F	CTGCGAGATGGCAGAGAAGTG	
CXCL1_R	GGCCTTGTCCAGAATTGTCTTG	
SAA_F	TGGGTCTGCATCGCATTG	
SAA_R	TGCATCCCGGACAAACTGT	
LEAP2_F	ATGCACTGGTGGAAAGTGA	
LEAP2_R	GACACTCCTCTCCAGAAG	
GAPDH_F	GGAGCGTGACCCCAGCAACA	
GAPDH_R	CACACGCTTGGCACCACCCT	

601



602

603 **Figure 1.** Phylogenetic and structural analysis of HfIL-1β. (A) A phylogenetic tree was
604 constructed using multiple alignments with amino acid sequences encoded precursor form of
605 HfIL-1β within MEGA 4 program. The clades were validated by 1.000 bootstrap replications,
606 which were represented by percentage in branch nodes. The scale bar represents a genetic
607 distance of 0.2. (B) Ribbon diagram of HfIL-1β and the superimposed HfIL-1β and ChIL-1β. A
608 ribbon diagram of the three-dimensional structure of HfIL-1β has shown (left). The α-helix and
609 β-strands indicate as helix and arrows, respectively, and the N- and C- termini are labeled. The
610 X-ray structure of HfIL-1β (yellow) is superimposed onto that of ChIL-1β (blue, right).



611

612 **Figure 2.** Immunoblot analysis of purified rHfIL-1β. (A) rHfIL-1β expressed from *E.coli* BL21
613 was detected with polyhistidine antibody. (B) Immunoblot analysis of purified rHfIL-1β was
614 performed using anti-ChIL-1β antibody, M, protein molecular weight marker (kDa); lane 1,
615 ChIL-1β (1 ??g) as a positive control; lane 2, purified rHfIL-1β (1 ??g).

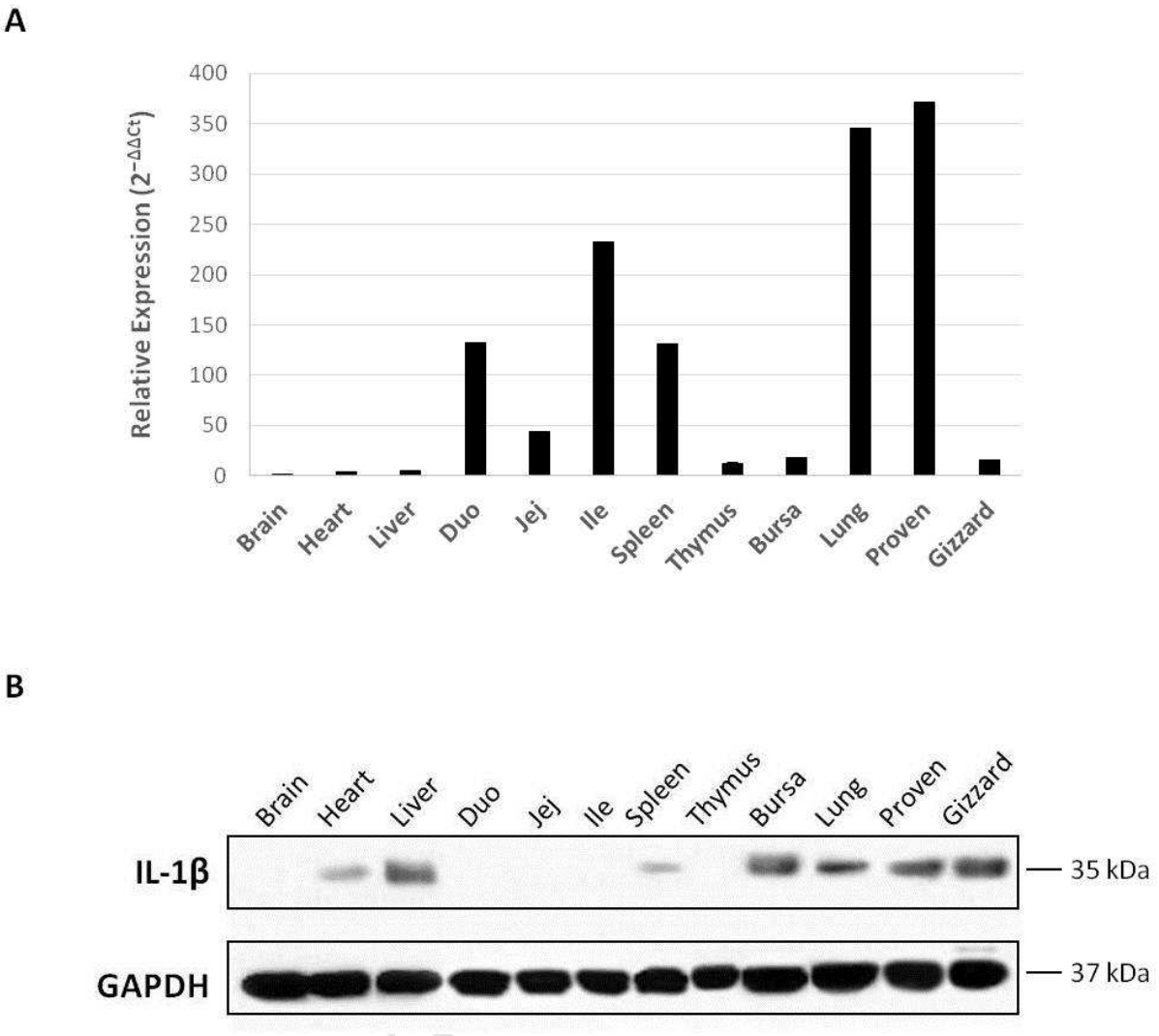


Figure 3. Expression pattern of mRNA and protein of HfIL-1β in various tissues of clinically healthy house finches. (A) mRNA expression of HfIL-1β in the different tissues was determined by qRT-PCR. Data was normalized to the expression level of GAPDH and represented as fold change relative to that of brain. Error bars indicate the SEM. (B) HfIL-1β protein expression from various healthy house finch tissues was assessed by immunoblotting using anti-ChIL-1β antibody, with GAPDH used as a loading control. (Duo, duodenum; Jej, jejunum; Ile, ileum; Proven, proventriculus)

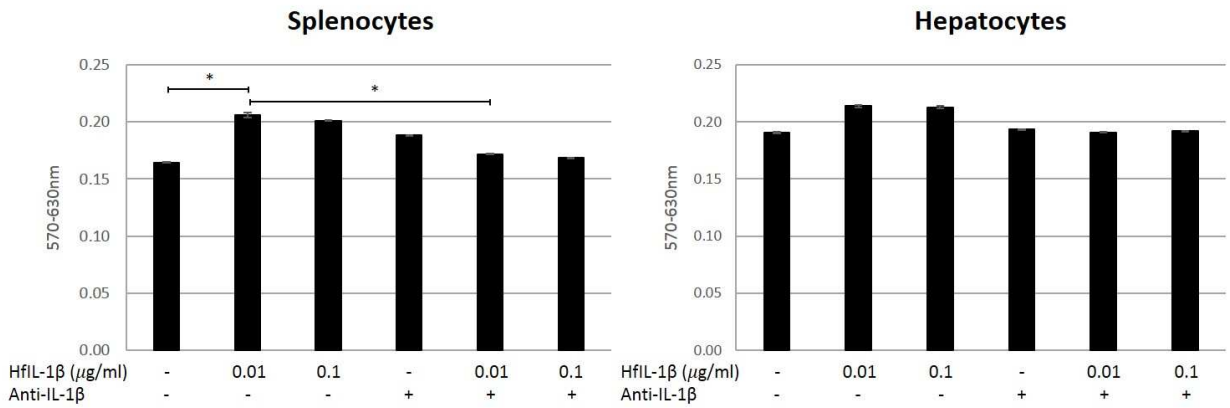
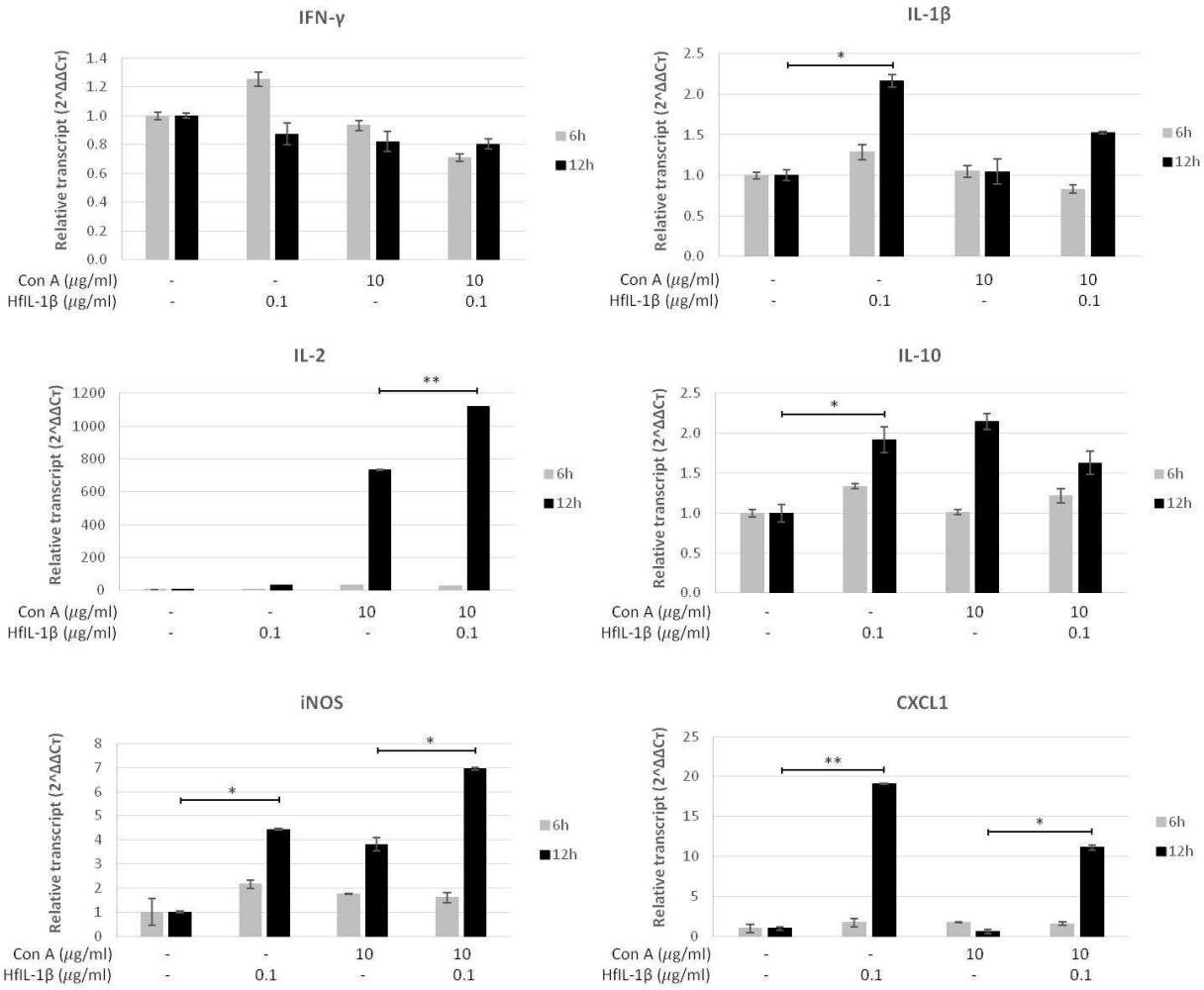


Figure 4. The effect of HfIL-1β on house finch cell proliferation in vitro. Splenocytes (2×10^5 cells/well) were incubated with medium alone, rHfIL-1β (0.01 and 0.1 ??g/ml), rHfIL-1β (0.01 and 0.1 ??g/ml) with anti-ChIL-1β antibody for 12 hr (left). Corresponding proliferation assay was conducted on hepatocytes (right). Anti-ChIL-1β antibody alone was used as a negative control. Data represent the mean ± SEM of two independent experiments performed in triplicate and asterisks indicate statistically significant differences ($p < 0.05$).



631

632 **Figure 5.** mRNA expression of Th1/Th2 cytokines and chemokine following stimulation of
633 splenocytes with HfIL-1β. Splenocytes (1×10^6 cells/well) were stimulated with medium alone,
634 rHfIL-1β (0.1 ??g/ml) alone, Con A (10 ??g/ml) alone, Con A plus rHfIL-1β (0.1 ??g/ml) for 6
635 and 12 hr. The expression of Th1/Th2 cytokines and a chemokine was evaluated by qRT-PCR.
636 Data are presented as the mean ± SEM of two independent experiments performed in triplicate.
637 Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$).

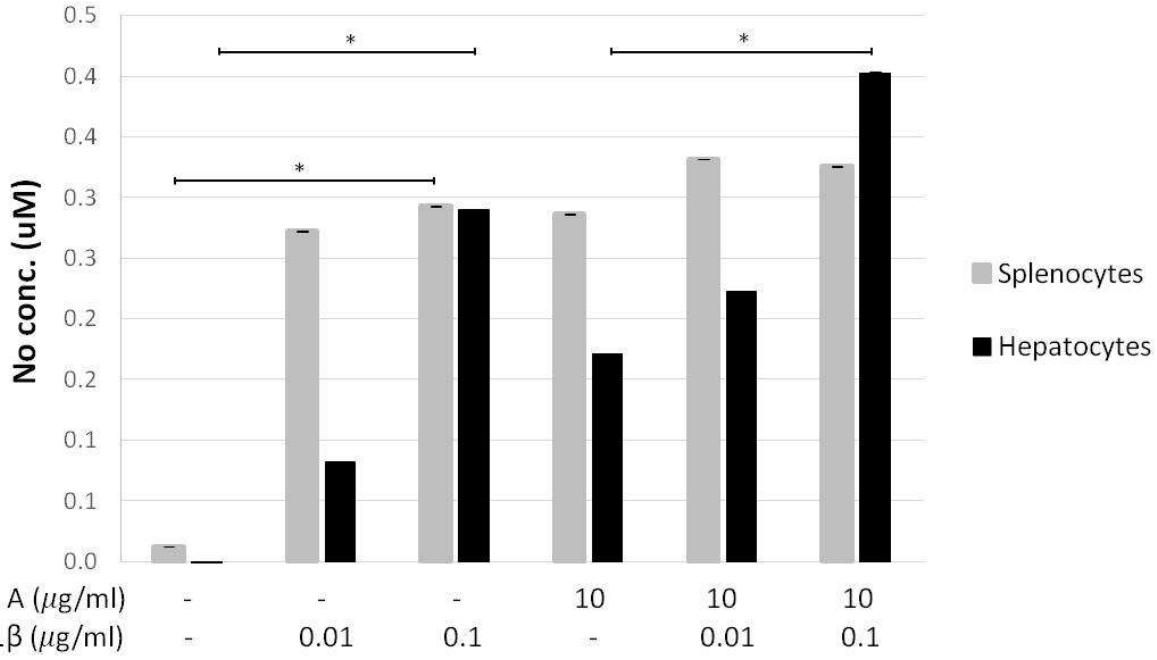


Figure 6. Nitric oxide release from HfIL-1β-stimulated splenocytes and hepatocytes. Splenocytes or hepatocytes (1×10^6 cells/well) were stimulated with medium alone, r HfIL-1β (0.01 and 0.1 ??g/ml) alone, Con A (10 ??g/ml) alone, Con A plus rHfIL-1β (0.01 and 0.1 ??g/ml) for 6 hr. The levels of NO were determined by Griess assay. Data are presented as the mean ± SEM of two independent experiments performed in triplicate and statistically significant difference indicated by asterisks ($p < 0.05$).

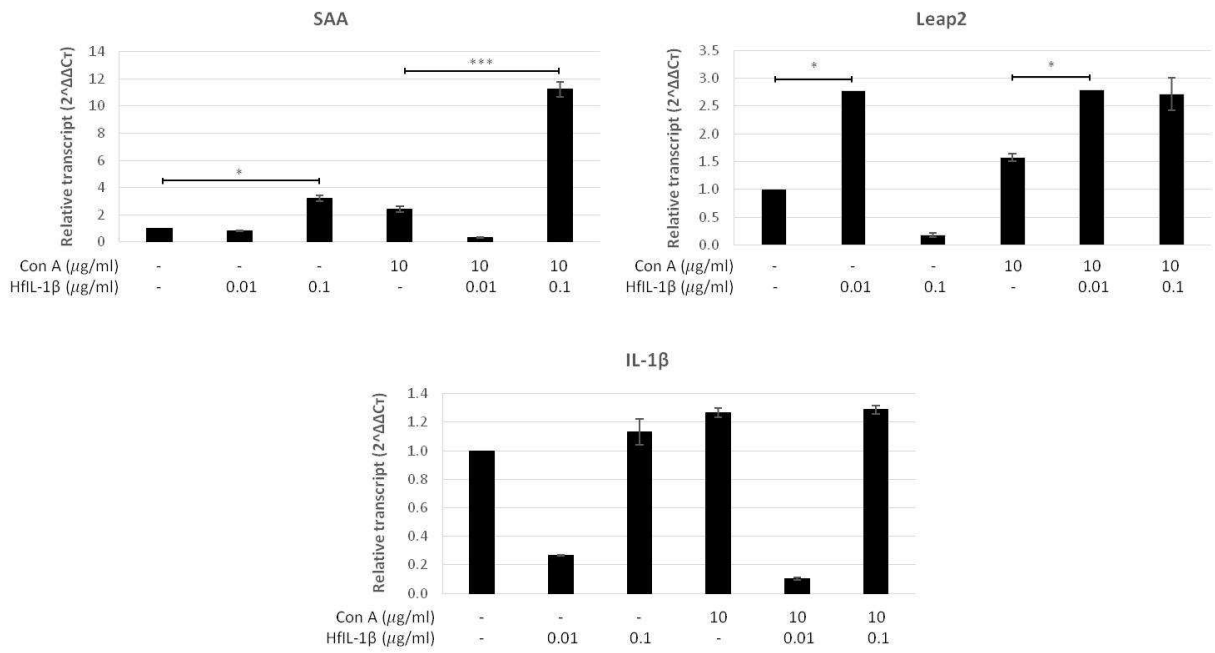


Figure 7. Production of acute phase protein and antimicrobial peptide by rHfIL-1β-stimulated hepatocytes. Hepatic cells (1×10^5 cells/well) were treated with medium alone, Con A (10 ??g/ml) alone, rHfIL-1β (0.01 and 0.1 ??g/ml) or rHfIL-1β (0.01 and 0.1 ??g/ml) with Con A for 6 hr. mRNA expression was measured with qRT-PCR and then values were normalized to GAPDH and graphed relative to medium alone. Data are presented as the mean ± SEM of two independent experiments performed in triplicate and significant differences indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Identification and functional characterization of the house finch interleukin-1β

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Highlights

- Full-length house finch IL-1β was cloned, expressed, and its basic biological roles explored.
- House finch IL-1β modulates the expression of Th1/Th2 cytokines and nitric oxide production by activated immune cells
- House finch IL-1β enhances the expression of acute phase protein and antimicrobial peptide by activated immune cells.